A role for calcium in the regulation of neutral trehalase activity in the fission yeast *Schizosaccharomyces pombe*

Alejandro FRANCO, Teresa SOTO, Jero VICENTE-SOLER, Vanessa PAREDES, Marisa MADRID, Mariano GACTO¹ and José CANSADO

Department of Genetics and Microbiology, Facultad de Biología, University of Murcia, 30071 Murcia, Spain

Neutral trehalases mobilize trehalose accumulated by fungal cells as a protective and storage carbohydrate. A structural feature of these enzymes is the presence of an EF-like motif similar to that shown by many Ca^{2+} -binding proteins. In this study we provide direct evidence for physical binding of Ca^{2+} to neutral trehalase (Ntp1p) of the fission yeast *Schizosaccharomyces pombe*, and show that aspartic residues at positions 97 and 108 in the conserved putative Ca^{2+} -binding motif of Ntp1p appear to be responsible for this interaction. Mutations in these residues do not interfere with the ability of Ntp1p to associate *in vivo* with trehalose-6-phosphate synthase, but prevent activation of neutral trehalase triggered by the addition of glucose or by subjecting cells to stressing conditions. Strains expressing Ntp1p variants that are unable to bind Ca^{2+} partially resemble those devoid of the *ntp1*⁺ gene in terms of trehalose hyperaccumulation. Gel filtration

of cell extracts from wild-type cells after EDTA treatment or from cells containing Ntp1p with mutations in aspartic acid residues within the Ca^{2+} -binding site revealed that Ntp1p eluted mainly in an inactive conformation instead of the dimeric or trimeric active form of the enzyme. These results suggest that activation of *S. pombe* Ntp1p under different conditions depends upon Ca^{2+} binding through the Ca^{2+} -binding motif as a prerequisite for correct enzyme oligomerization to its active form. Given the high degree of conservation of the Ca^{2+} accommodation site, this might be a general mechanism regulating neutral trehalase activity in other yeasts and filamentous fungi.

Key words: calcium binding, EF hand, enzyme oligomerization, neutral trehalase activity, *Schizosaccharomyces pombe*.

INTRODUCTION

The non-reducing disaccharide trehalose (α -D-glucopyranosyl- α -D-glucopyranoside) plays an important role as a reserve and stress metabolite in yeast cells and filamentous fungi [1,2]. The ability of this storage carbohydrate to protect against denaturation of proteins and other cellular components *in vitro* correlates with a protective function against various environmental stresses *in vivo* [3,4]. Several studies have shown that accumulation of this sugar induced by either sublethal stresses or overexpression of genes responsible for trehalose synthesis affords greater survival to yeast cells under extreme conditions [5,6]. Conversely, other studies have demonstrated that mutant cells deficient in trehalose biosynthesis or with increased expression of enzymes able to hydrolyse this sugar display decreased resistance against various stresses [7]. The preservation properties of trehalose rely on the possible physical interaction of the hydroxy groups of this sugar with polar residues of cellular components, as explained by the water replacement hypothesis [8]. However, efficient renaturation of partially unfolded proteins by molecular chaperones after stress is inhibited by high levels of trehalose, so the protective process requires effective removal of trehalose during recovery [9].

The enzymic hydrolysis of trehalose is carried out by trehalases, two basic types of which are known to exist in the fungal world on the basis of their pH optima: acid and neutral trehalases [2]. These two enzyme species do not share relevant structural identity and have distinct functions. Acid (or non-regulatory) trehalases have been localized to the vacuole or to the cell wall, and appear to be required for growth on trehalose as a carbon source [10]. Neutral (or regulatory) trehalases are cytoplasmic enzymes

able to mobilize the endogenous trehalose pool whose enzyme activity is modulated by metabolic signals triggered by nutrients and physical stresses [11,12]. The co-existence of neutral trehalases and their substrate trehalose within the cytosol suggests a fine regulatory mechanism to allow proper trehalose breakdown in response to extracellular stimuli. Neutral trehalases can thus be used as intracellular probes to outline pathways involved in the transmission of regulatory signals and to define the metabolic status of fungal cells. Several lines of evidence indicate that neutral trehalase activity is controlled by enzyme phosphorylation through a mechanism that involves PKA (cAMP-dependent protein kinase) [12,13], and that the active form of these trehalases corresponds to dimers or trimers of the enzyme protein [14,15].

Neutral trehalases share a common C-terminal domain that is likely to contain the catalytic core of the enzyme [16,17]. It has been proposed that a large N-terminal extension forms a regulatory domain, because of the occurrence in this portion of at least one perfect consensus site for PKA-mediated phosphorylation [11,16–20]. Another molecular feature of neutral trehalases that has been characterized is the presence in the N-terminal domain of a Ca^{2+} -binding site that is highly similar to well known Ca^{2+} -binding consensus sequences that are present in other proteins [16–18,20,21]. In this context, various studies support the involvement of Ca^{2+} in the activity of fungal neutral trehalases, because these enzymes are known to require Ca^{2+} or Mn^{2+} for activity and to be inhibited by EDTA [14,16].

Studies attempting to define the contribution of the phosphorylation sites to the activation of neutral trehalases have been performed by using *Saccharomyces cerevisiae* mutants altered in specific residues of evolutionarily conserved putative

Abbreviations used: GST, glutathione S-transferase; Ha, haemagglutinin; Ha6H epitope, epitope comprising haemagglutinin antigen plus six histidine residues; IPTG, isopropyl β -D-thiogalactoside; ORF, open reading frame; PKA, cAMP-dependent protein kinase.

¹ To whom correspondence should be addressed (e-mail maga@um.es)

Table 1 *S. pombe* strains used in this studyAll strains are *ade6*-M216 *leu1*-32 *ura4*-D18.

Strain	Genotype	Source or reference
MM1	<i>h</i> ⁺	Laboratory stock
MMT-3	<i>h</i> ⁺ <i>ntp1::ura4</i> ⁺	[20]
N1	<i>h</i> ⁺ <i>ntp1::ura4</i> ⁺ <i>ura4::ntp1⁺:Ha6H</i>	This study
N7	<i>h</i> ⁺ <i>ntp1::ura4</i> ⁺ <i>ura4::ntp1⁺-D97L:Ha6H</i>	This study
N8	<i>h</i> ⁺ <i>ntp1::ura4</i> ⁺ <i>ura4::ntp1⁺-R100L:Ha6H</i>	This study
N9	<i>h</i> ⁺ <i>ntp1::ura4</i> ⁺ <i>ura4::ntp1⁺-I104L:Ha6H</i>	This study
N10	<i>h</i> ⁺ <i>ntp1::ura4</i> ⁺ <i>ura4::ntp1⁺-D108L:Ha6H</i>	This study
C4	<i>H</i> ⁻ <i>tps1⁺:GST (ura4⁺)</i>	[15]
N1TG	<i>h</i> ⁺ <i>ntp1::ura4</i> ⁺ <i>ura4::ntp1⁺:Ha6H</i> <i>tps1⁺:GST(ura4⁺)</i>	This study
N7TG	<i>h</i> ⁺ <i>ntp1::ura4</i> ⁺ <i>ura4::ntp1⁺-D97L:Ha6H</i> <i>tps1⁺:GST(ura4⁺)</i>	This study
N8TG	<i>h</i> ⁺ <i>ntp1::ura4</i> ⁺ <i>ura4::ntp1⁺-R100L:Ha6H</i> <i>tps1⁺:GST(ura4⁺)</i>	This study
N9TG	<i>h</i> ⁺ <i>ntp1::ura4</i> ⁺ <i>ura4::ntp1⁺-I104L:Ha6H</i> <i>tps1⁺:GST (ura4⁺)</i>	This study
N10TG	<i>h</i> ⁺ <i>ntp1::ura4</i> ⁺ <i>ura4::ntp1⁺-D108L:Ha6H</i> <i>tps1⁺:GST (ura4⁺)</i>	This study

phosphorylation sites [22]. However, in spite of the evidence for a Ca²⁺-dependent neutral trehalase activity, the functionality of the EF-like Ca²⁺-binding site present in fungal neutral trehalases remains to be established. EF-sequence motifs are typically found in pairs in many proteins able to link Ca²⁺ [23], whereas only one is present in fungal neutral trehalases [16,17,20].

Many essential cellular processes, such as cell cycle control, nucleotide metabolism and signal transduction, are tightly regulated by calcium. In numerous Ca²⁺-binding proteins, regulatory domains undergo large conformational changes upon ion binding, and this response modulates their overall intramolecular conformation and their interaction with other proteins, including aggregation and dissociation [23–25]. The present study reveals that Ntp1p (neutral trehalase) from *Schizosaccharomyces pombe* is Ca²⁺-dependent. We suggest that this ion is likely to be an integral part of active trehalase, and demonstrate that its binding to the EF-like consensus site is required not only for full activity *in vitro* but also for stress-induced activation of the enzyme *in vivo*. Site-directed mutagenesis indicates that the effects of Ca²⁺ are specifically mediated by this motif, whose alteration promotes changes in the oligomerization pattern of the enzyme which appear to be required for functional trehalase activity.

EXPERIMENTAL

Yeast strains and growth conditions

The *S. pombe* strains employed in this study are listed in Table 1. Cells were routinely grown with shaking at 28 °C in YES (rich) or EMM2 (minimal) medium [26] supplemented with adenine, leucine, histidine or uracil (100 mg · l⁻¹) depending on the requirements for each particular strain. 5-Fluoro-orotic acid medium was used to isolate homologous insertions of Ntp1p–Ha (haemagglutinin) constructs into the *ura4*⁺ locus [27]. Mating of strains was performed in MEL medium [26], and the purified ascospores were grown in EMM2 medium with the appropriate supplements. Solid media were made by the addition of 2% (w/v) bacto-agar.

Plasmid constructs, site-directed mutagenesis and *S. pombe* transformation

Plasmid pBura4 contains the *ura4*⁺ gene cloned as two separate *Xba*I–*Bam*HI (950 bp) and *Kpn*I–*Xho*I (850 bp) fragments into

Table 2 Oligonucleotide pairs used for site-directed mutagenesis experiments at the *ntp1*⁺ ORFBase changes relative to the *ntp1*⁺ wild-type sequence are indicated in boldface.

Name	Sequence (5' to 3')	Mutation
D97L5	GGAATCTGAAG GC ACCGACCGG	D97L
D97L3	CCGGTCGGTG GC TTTCAGATTCC	
R100L5	GACACCGAC CT GAACATGCAATC	R100L
R100L3	GATTTCATGTT CA GGTCGGTGTC	
I104L5	GAACATGCA ACT CACCATCGAGG	I104L
I104L3	CCTCGATGGT GAG TTGCATGTTTC	
D108L5	CACCATCGAG GC TCGACCGGACC	D108L
D108L3	GGTCCGGTG CA GCCTCGATGGTG	

the pBluescript SKII⁺ plasmid. Plasmid pBura4-*ntp1*⁺:*Ha6H* contains the *ntp1*⁺ gene fused at its C-terminus with an Ha6H epitope (epitope comprising haemagglutinin antigen plus six histidine residues) plus 1.1 kb of the promoter region cloned as a 4.1 kb *Xho*I–*Bam*HI fragment into pBura4, and flanked by *ura4*⁺ fragments. The mutations at Asp⁹⁷, Arg¹⁰⁰, Ile¹⁰⁴ and Asp¹⁰⁸ residues in Ntp1p were created by the overlap extension method with the use of PCR [28]. Two separate amplification reactions were performed with plasmid pBura4-*ntp1*⁺:*Ha6H* as template with the use of a first pair of primers [PRO52 (TCCGCTCGAGATCGGTTAGTTCAGAGTC; *Xho*I site is underlined) and the corresponding 5' mutagenic oligonucleotide (D97L5, R100L5, I104L5 or D108L5; see Table 2)], and a second pair of primers [NTP3M (ACTGGCATCGATTCTTCGAGT; *Cla*I site is underlined) and the corresponding 3' mutagenic oligonucleotide (D97L3, R100L3, I104L3 or D108L3; Table 2)]. The two PCR products were purified by agarose gel electrophoresis, mixed, and subjected again to PCR with primers PRO52 and NTP3M. PRO52 and NTP3M hybridize at positions –1092 to –1075 in the *ntp1*⁺ promoter and at positions +541 to +561 in the *ntp1*⁺ ORF (open reading frame) respectively. The resultant 1.7 kb fragments were digested with *Xho*I and *Cla*I and cloned into pBura4-*ntp1*⁺:*Ha6H*, creating plasmids pBura4-*ntp1*⁺-D97L, pBura4-*ntp1*⁺-R100L, pBura4-*ntp1*⁺-I104L and pBura4-*ntp1*⁺-D108L, which contain the promoter plus the different mutated versions of *ntp1*⁺ fused to Ha6H and flanked by *ura4*⁺ sequences. These plasmids, together with pBura4-*ntp1*⁺:*Ha6H* (wild-type Ntp1p), were digested with *Xba*I and *Kpn*I, and the linear fragments were separately integrated in single copy into the *ura4*⁺ locus of *S. pombe* strain MMT-3 (Δ *ntp1*) by transforming cells to 5-fluoro-orotic acid resistance as previously described [27]. The homologous integration of the constructs into *ura4*⁺ was verified by Southern blot analysis.

Immunoprecipitation of Ha6H- and GST (glutathione S-transferase)-tagged proteins

For immunoprecipitation of Ha6H-tagged proteins, cell extracts were incubated for 12 h at 4 °C with monoclonal mouse anti-Ha antibody (clone 12CA5; Roche Molecular Biochemicals), and the immunocomplexes were adsorbed with Protein A–agarose (Roche Molecular Biochemicals) for 4 h at 4 °C. The immunoprecipitation of GST-tagged proteins was performed with a sheep anti-GST polyclonal antibody (Amersham-Pharmacia) and Protein G–agarose (Roche Molecular Biochemicals). In all cases the suppliers' recommendations were followed in terms of incubation times and washing of the complexes.

SDS/PAGE and Western blotting

Proteins were resolved in 10% (w/v) acrylamide SDS/PAGE gels as previously described [15], transferred to nitrocellulose filters (Amersham-Pharmacia) and incubated with mouse anti-Ha or sheep anti-GST antibodies. The immunoreactive bands were revealed with anti-mouse or anti-sheep horseradish peroxidase-conjugated secondary antibodies (Sigma Chemical Co.) and the ECL[®] system (Amersham-Pharmacia).

Gel filtration

When required, desalting of enzyme preparations was carried out in Sephadex G25 columns (Pharmacia) using buffer A (10 mM Mes, pH 6.0) as solvent, and the fractions corresponding to the void volume were used in enzyme assays. For studies on size-exclusion analysis, a Superdex-200 column (Amersham-Pharmacia) equilibrated with buffer A supplemented with 150 mM NaCl was used in an AKTA HPLC system (Amersham-Pharmacia). Lower salt concentrations were not used, in order to minimize non-specific electrostatic interactions with the column matrix. The column was calibrated using vitamin B₁₂ (1.3 kDa), cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), BSA (66 kDa), yeast alcohol dehydrogenase (150 kDa), β -amylase (200 kDa), apoferritin (430 kDa) and thyroglobulin (670 kDa) (all from Sigma Chemical Co.) at the concentrations recommended by the manufacturer. Aliquots of 100 μ l (1 mg of total protein) of the supernatant from ultracentrifuged extracts obtained from Ha6H-tagged strains N1 or N7 grown to mid-exponential phase, or after treatment with 0.5 M EDTA, were applied to the column with buffer A plus a yeast protease inhibitor cocktail (Sigma Chemical Co.). A flow rate of 0.4 ml/min was used and the elution was tracked by absorbance at 280, 235 and 220 nm. Blue Dextran (2000 kDa; Sigma) and vitamin B₁₂ were used for determination of void volume and total bed volume respectively. Molecular mass values were estimated from a calibration curve (correlation coefficients ≥ 0.98) generated by linear regressions of the elution volume for each protein using SigmaPlot (Jandel Scientific). To determine the fractions containing Ntp1p-Ha6H protein, 100 μ l of each fraction (0.4 ml) was precipitated with trichloroacetic acid, washed with cold acetone, air-dried, resuspended in SDS gel sample buffer, and resolved in SDS/PAGE (10%) gels. Immunoreactive bands were detected by Western blot analysis with anti-Ha antibodies.

Enzyme assays, trehalase activation and trehalose content

Trehalase activity was assayed after cell breakage as described previously [29], and expressed as units per mg of protein. Activation of trehalase by the addition of glucose (0.1 M) was performed in stationary-phase cultures after growth in YES medium [29]. For stress-induced trehalase activation, cultures were grown in YES medium to mid-exponential phase ($D_{600} = 0.7$ – 1.0) and then subjected to either heat treatment (40 °C) or osmotic upshift (0.75 M NaCl) for different times, depending on the experiment [30,31]. Intracellular trehalose was extracted and estimated as reported by Beltrán et al. [32]. All determinations were repeated at least three times with consistent results. Unless otherwise indicated, representative results are shown.

Expression and purification of Ntp1p in *Escherichia coli*

The wild-type and mutated versions of the *ntp1*⁺ ORF fused to the Ha6H epitope were amplified by PCR using plasmids pBura4-*ntp1*⁺-Ha6H, pBura4-*ntp1*⁺-D97L, pBura4-*ntp1*⁺-R100L, pBura4-*ntp1*⁺-I104L and pBura4-*ntp1*⁺-D108L

Table 3 Effect of calcium-binding chelators on trehalase activity in cell-free extracts from exponential-phase cultures of *S. pombe* strain MM1

Results are means $\pm$ S.E.M. ($n \geq 6$)

Additions	Trehalase activity (units/mg of protein)	
	Unstressed cells	Heat-shocked cells (40 °C, 1 h)
None	1.9 $\pm$ 0.15	19.1 $\pm$ 1.69
5 mM Ca ²⁺	1.7 $\pm$ 0.15	19.0 $\pm$ 1.40
5 mM Mn ²⁺	2.1 $\pm$ 0.20	20.3 $\pm$ 1.75
10 mM EGTA	0.3 $\pm$ 0.01	2.8 $\pm$ 0.15
10 mM EGTA, then 20 mM Ca ²⁺	< 0.1	0.2 $\pm$ 0.01
10 mM EDTA	0.2 $\pm$ 0.01	0.7 $\pm$ 0.04
10 mM EDTA, then 20 mM Ca ²⁺	< 0.1	0.2 $\pm$ 0.01

as templates, with the 5' oligonucleotide *BACT5* (GAT-CCATGGGAATGCCTTCGAAATTTTCC; *Nco*I site is underlined) and the 3' oligonucleotide *BACT3* (CCGCTCGAGTT-AGTGGTGATGATGGTG; *Xho*I site is underlined). The resulting 2.5 kb fragments were digested with *Xho*I and *Nco*I, and cloned under the control of T7 promoter into plasmid pET-15b, an *E. coli* expression vector (Novagen). The resulting plasmids were transformed into *E. coli* BL21. The strains were grown in LB medium [33] plus ampicillin (50 μ g/ml) in the presence of IPTG (isopropyl β -D-thiogalactoside) for 4 h and, once the corresponding cleared lysates were obtained, the Ntp1p-Ha6H fusions were purified using Ni²⁺/nitriloacetic acid-agarose beads following the procedures recommended by the supplier (Qiagen Inc.)

⁴⁵Ca²⁺ overlay assay

After Ni²⁺ purification, 10 μ g of each of the Ntp1p-Ha6H proteins was dot-blotted on to a nitrocellulose membrane and the blots were washed extensively for 4 $\times$ 15 min with wash buffer (10 mM imidazole/HCl, pH 7.6, 70 mM KCl, 0.5 mM MgCl₂). A ⁴⁵Ca²⁺ overlay assay [34] was performed by incubating the blots with 3 μ M ⁴⁵CaCl₂ (10 mCi/l; NEN) for 12 h at 20 °C, followed by consecutive washes with deionized water and 50% (v/v) ethanol. After the blot was air-dried, it was exposed to Hyperfilm (Amersham Biosciences) for 48 h. Positive, calcium-binding controls included calmodulin (Sigma) and calpain (Sigma), with BSA (Roche) and alcohol dehydrogenase (Sigma) employed as negative controls.

RESULTS

Effect of Ca²⁺ on trehalase activity *in vitro*

Neutral trehalase activity from the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* responds *in vivo* to several environmental changes, and is strongly activated by Ca²⁺ or Mn²⁺ *in vitro* [12,14,16]. In this context, we reported previously that the neutral trehalase from *S. pombe* (Ntp1p) is also activated *in vivo* by various stresses, and showed a bivalent ion requirement *in vitro*, since the addition of EDTA to cell extracts greatly decreased trehalase activity [20]. Data summarized in Table 3 indicate that the addition of CaCl₂ to cell extracts obtained from exponential-phase cells of the fission yeast either growing at 28 °C or after a heat-shock treatment did not significantly enhance the values of basal trehalase activity in each case. Although the results given in Table 3 correspond only to one particular Ca²⁺

88	-TLNALLESEDTRNMQITIEDTGPKVVSLS-	118	<i>S. pombe</i>
105	-TLNELLAEDTDKNYQITIEDTGPKVLKVG-	135	<i>S. cerevisiae</i> (NTH1)
96	-TLHSLLESDSDRNMQITIEDVGPKVTAAS-	126	<i>E. nidulans</i>
135	--LDELLASEDTKNNHQITIEDTGPKVIKVG-	164	<i>S. cerevisiae</i> (NTH2)
108	-TLKELLASEDTGNYQITIEDTGPKVIRVG-	138	<i>K. lactis</i>
233	-TLRELLANEDTDNNCQITIEDTGPKVRLGT-	263	<i>C. albicans</i>
90	-TLQSLQSQEDTDGNMQITIEDNGPKVLSL---	118	<i>N. crassa</i>
83	-TLESLQAQEDTDGNMQITIEDEGPKVLSL---	111	<i>M. anisopliae</i>
83	-TLESLKQSQEDTDGNMQITIEDNGPKVLT---	111	<i>M. grisea</i>
Consensus --L--L---EDTD-N-QITIED-GPKV+-----			
Consensus (Fungal/yeast trehalases) D - T - D - X - N - X - Q - I - T - I - E - D			
Consensus (PROSITE EF Hand signature) D - X - D - I - D - D - G - L - D - X - X - D			
	N L E N P I E		
	S V N Q V N		
	F S G M Q		
	Y T H C S		
	W G R T		
	K		
		A	
		G	
		C	

Figure 1 Conservation of a putative calcium-binding motif in neutral trehalases from fungi

Shown is a multiple sequence alignment of predicted amino acid sequences of the putative calcium-binding motifs in neutral trehalases from *Schizosaccharomyces pombe* [20], *Saccharomyces cerevisiae* (NTH1 [11]), *Emergella (Aspergillus) nidulans* [17], *Saccharomyces cerevisiae* (NTH2; putative trehalase protein [19]), *Kluyveromyces lactis* [16], *Candida albicans* [18], *Neurospora crassa* [17], *Metarhizium anisopliae* [39] and *Magnaporthe (Pyricularia) grisea* [38]. Amino acids are shown in single-letter code. Identical amino acids in the consensus sequence are presented in bold. The PROSITE consensus sequence (database reference PDOC000018) for the calcium-binding loop in proteins with the EF-hand motif is also shown.

concentration, we were equally unsuccessful in demonstrating activation in a range from 0.01 to 10 mM CaCl_2 . Similar results were obtained for Mn^{2+} (not shown). However, the presence of EDTA or of the more specific Ca^{2+} -chelating agent EGTA did result in markedly low trehalase activity. These data confirm that Ntp1p is strongly dependent on $\text{Ca}^{2+}/\text{Mn}^{2+}$ for activity. Further addition of salts containing $\text{Ca}^{2+}/\text{Mn}^{2+}$ to EDTA- or EGTA-treated enzyme preparations did not prompt recovery of the enzyme activity. Instead, sequential inhibition was produced by these supplements in assays attempting re-activation (Table 3). Desalting of EGTA-treated enzyme preparations on a Sephadex G25 column also failed to show trehalase re-activation by Ca^{2+} (results not shown). These observations are compatible with the interpretation that once $\text{Ca}^{2+}/\text{Mn}^{2+}$ is removed from the enzyme protein by chelating compounds, an essentially irreversible denaturation occurs, so that the resulting enzyme is unable to be re-activated upon further $\text{Ca}^{2+}/\text{Mn}^{2+}$ addition.

A detailed analysis of the amino acid sequence of Ntp1p revealed the presence in the N-terminal region of a putative calcium-binding site (sequence DTDNRNMQITIED; Figure 1) whose existence was first described in neutral trehalases from *Saccharomyces cerevisiae* and *K. lactis* [16]. This site appears to be conserved in all yeast and fungal neutral trehalases so far analysed (Figure 1). Such a sequence is very similar to the calcium-binding site present in the helix-loop-helix EF-hand motif of numerous calcium-binding proteins, such as calmodulin, calcineurin B and troponin C [23,35]. In all cases, the acidic residues at positions 1 and 12 in the motif are known to directly co-ordinate Ca^{2+} ions, whereas residues at positions 3, 5 or 9 may also be involved, depending on the particular protein (Figure 1; [23]). Hence we considered it very likely that this site might play an important role in the activity of the neutral trehalase in fission yeast.

Construction of strains that express Ntp1p mutated in different residues of the putative calcium-binding motif

Our first goal in attempts to assess the biological significance of the conserved putative calcium-binding site in neutral trehalase

activation was to construct *S. pombe* strains containing versions of the *ntp1⁺* gene mutated in different residues of the motif. We performed site-directed mutagenesis at the *ntp1⁺* ORF and generated DNA constructs to express the wild-type (control) and D97L, R100L, I104L and D108L variants of Ntp1p fused to an Ha6H epitope at their C-terminus (see the Experimental section). We chose to mutagenize the conserved aspartic acid residues at positions 97 and 108 because these acidic amino acids, at positions 1 and 12 within the EF-hand motif, are critical for bivalent ion binding by proteins that harbour such a binding site [23]. On the other hand, arginine at position 100 is not a conserved residue, whereas isoleucine at position 104 (position 8 within the sequence; Figure 1) appears to be important for the correct conformation of the motif, although not directly involved in ion binding [23]. These constructs were integrated in single copy at the *ura4⁺* locus in strain MMT-3, which is a mutant lacking neutral trehalase [7], and the uracil auxotrophs were selected for 5-fluoroorotic acid resistance. The correct integration of the constructs was then confirmed by Southern blot (not shown). Thereafter, we performed a Western blot analysis with anti-Ha antibodies and cell extracts obtained from both the control strain (N1; non-mutated Ntp1p) and the Ntp1p-mutated strains N7 (D97L), N8 (R100L), N9 (I104L) and N10 (D108L) growing to mid-exponential phase, before and after a heat or osmotic stress. As shown in Figure 2, all strains displayed similar levels of expression of the Ntp1p-Ha fusion protein, with a clear increase in protein synthesis upon heat or osmotic stress. These results validated the usefulness of these strains as a tool for studying the effects of the specific mutations described in trehalose breakdown by fission yeast.

Aspartic acid residues in the putative calcium-binding motif of Ntp1p are essential for activation of neutral trehalase

If the conserved calcium-binding site present in Ntp1p plays a major role in enzyme activation, then mutations in the main residues of this motif should greatly affect neutral trehalase activity under different conditions. We tested this hypothesis by measuring neutral trehalase activity at different times after

Figure 2 Ntp1p levels in *S. pombe* strains expressing different neutral trehalase mutants

Western blot analysis of Ntp1p-Ha6H synthesis in *S. pombe* strains N1 (control), N7 (D97L), N8 (R100L), N9 (I104L) and N10 (D108L) was performed employing anti-Ha antibodies. Exponentially growing cells were analysed before stress treatment (lane B), and after a heat shock (40 °C for 60 min; lane T) or osmotic stress (0.75M NaCl for 60 min; lane O). Incubation with anti- α -tubulin was used as a loading control (lower panel).

Figure 3 Key residues in the putative calcium-binding motif of Ntp1p involved in activation of neutral trehalase

At zero time, cultures of N1 (control; Δ), N7 (D97L; $\blacksquare$), N8 (R100L; $\square$), N9 (I104L; $\bullet$) and N10 (D108L; $\blacktriangle$) strains were pulsed with 100 mM glucose (A), shifted to 40 °C (B) or subjected to osmotic stress by addition of 0.75 M NaCl (C). Neutral trehalase activity was measured in cell samples at the times indicated and compared with that in control cultures without treatment ($\circ$). Results are means $\pm$ S.E.M. of three determinations.

addition of 100 mM glucose to stationary-phase cultures of the control strain N1 and the Ntp1p mutant strains N7 (D97L), N8 (R100L), N9 (I104L) and N10 (D108L) (Figure 3A). Addition of glucose to cells growing on a non-fermentable carbon source, stationary-phase cells and ascospores is known to induce activation of neutral trehalase in this and other yeasts [2,29]. Alternatively, mid-exponential-phase cultures of the same strains were subjected to thermal (Figure 3B) or osmotic (Figure 3C) upshifts, which also induce strong trehalase activation in wild-type *S. pombe* cells [30,31]. As shown in Figure 3, aspartic acid residues at positions 97 and 108 (N7 and N10 strains) were critical for Ntp1p activation under all the conditions tested, whereas mutant R100L cells (N8 strain) displayed a slight increase in enzyme activity under heat shock (Figure 3B), but practically none after the glucose pulse or during osmotic stress (Figures 3A and 3C). Finally, cells with the I104L mutation (N9 strain) showed levels of neutral trehalase activation similar to those of control strain N1 under the same conditions (Figures 3A–3C), which is the typical response of wild-type strains [29–31]. These results demonstrate that, in *S. pombe* Ntp1p, mutations in aspartic acid residues at positions 97 and 108 within the putative bivalent ion-binding site bring about a drastic change that completely prevents neutral trehalase activation under all conditions assayed. In contrast, alterations in residues that are less significant for Ca^{2+} binding either still allow activation to a lesser extent under heat shock (R100L) or have no effect whatsoever on the activation response (I104L).

Figure 4 Trehalose content in *S. pombe* Ntp1p mutant strains upon stress

Mid-exponential-phase cells of MM1 (wild type), N1 (control), N7 (D97L), N8 (R100L), N9 (I104L), N10 (D108L) and MMT-3 ($\Delta ntp1$) strains were maintained in culture without treatment ($\square$), or subjected for 90 min ($\blacksquare$) to (A) osmotic stress (0.75 M NaCl) or (B) thermal stress (40 °C). Data represent means of three independent experiments; error bars indicate S.D.s.

Trehalose levels in *S. pombe* containing Ntp1p with mutations in the putative calcium-binding motif

Part of the survival strategy of *S. pombe* involves a rise in the intracellular trehalose content when cells are subjected to stress, with this storage disaccharide acting as a protectant metabolite against adverse environmental conditions [5,30]. Due to their inability to hydrolyse trehalose, *S. pombe* strains lacking the *ntp1*⁺ gene, which codes for neutral trehalase, show a higher trehalose content than control cells after a heat or osmotic stress [7]. A corollary of the results shown in Figure 3 is that the phenotype of Ntp1p mutant strains N7 (D97L) and N10 (D108L) should be similar to that of $\Delta ntp1$ strains, because of their inability to activate trehalase under any conditions. Consequently, we tested whether cells of these strains, like $\Delta ntp1$ cells, accumulate more trehalose than control cells (N1). Figure 4 shows results from the analysis of the trehalose content in wild-type strain MM1 and in strains N1 (control), N7, N8, N9, N10 and MMT-3 ($\Delta ntp1$), in exponentially growing cultures and after stress. Congruent with the above proposal, strains N7, N8 (R100L) and N10 accumulated as much trehalose as the *ntp1*⁺-deficient strain MMT-3 during osmotic stress (Figure 4A). In contrast, strains N1 (control) and N9 (I104L), which displayed strong neutral trehalase activation under the same conditions (Figure 3C), accumulated trehalose to wild-type levels. However, under thermal stress, the results were not as conclusive, although they followed a similar trend (Figure 4B). Strains N1 (control) and N9 accumulated lower levels of trehalose compared with strain MMT-3 after a heat shock, whereas the trehalose content in strains N7 (D97L), N8 (R100L) and N10 (D108L), which showed decreased trehalase activation under the same conditions, was above the control value but lower than that found in the *ntp1*⁺-disrupted mutant (Figure 4B). Taken together, these results highlight that *S. pombe* strains carrying versions of Ntp1p mutated in amino acid Asp⁹⁷ or Asp¹⁰⁸ resemble to some extent those devoid of the *ntp1*⁺ gene in terms of trehalose accumulation during osmotic stress and, less clearly, under thermal stress.

Figure 5 Co-immunoprecipitation of Ntp1p and Tps1p

The double-tagged strains N1TG (Ntp1p–Ha6H, Tps1p–GST; lane 2), N7TG (Ntp1p–D97L–Ha6H, Tps1p–GST; lane 3), N8TG (Ntp1p–R100L–Ha6H, Tps1p–GST; lane 4), N9TG (Ntp1p–I104L–Ha6H, Tps1p–GST; lane 5) and N10TG (Ntp1p–D108L–Ha6H, Tps1p–GST; lane 6) were grown to mid-exponential phase, subjected to a heat stress (40 °C, 60 min), and Tps1p–GST and Ntp1p–Ha6H variants were immunoprecipitated from the corresponding extracts with anti-GST (A) or anti-Ha (B) antibodies. After incubation with Protein A–agarose (anti-Ha immunoprecipitations) or Protein G–agarose (anti-GST immunoprecipitations) and extensive washing, the immunocomplexes were resolved by SDS/PAGE and analysed by Western blot using anti-Ha (A) or anti-GST (B) antibodies. Lane 1 in each panel corresponds to negative controls without immunoprecipitation, but incubated with Protein G–agarose (A) or Protein A–agarose (B) to account for possible non-specific protein binding to the matrix.

Mutations affecting the putative Ntp1p calcium-binding domain do not alter the Ntp1p–Tps1p interaction

Previous work has demonstrated that Ntp1p and Tps1p (trehalose-6-phosphate synthase) interact *in vivo* to form reversible protein complexes, and that the active form of neutral trehalase is associated with Ntp1p dimers or trimers, but not with complexes containing Tps1p [15]. In this context, other reports have shown that the presence of Tps1p is necessary for neutral trehalase activation by glucose or heat stress, but not during osmotic stress [5,36], raising the possibility that heat shock activates trehalase through a specific mechanism involving the prior formation of Tps1p–Ntp1p complexes. Because neutral trehalase mutated at Asp⁹⁷ or Asp¹⁰⁸ is not activated by heat stress (Figure 3B), we investigated whether this phenotypic trait could be due to a lack of interaction between Ntp1p and Tps1p. We constructed strains N1TG, N7TG, N8TG, N9TG and N10TG by mating strain C4 (containing Tps1p–GST) with strains N1, N7, N8, N9 and N10 respectively (Table 1). The new strains express Ntp1p (in wild-type form or the corresponding mutated version) and Tps1p fused at their C-termini to Ha6H and GST epitopes respectively, and their synthesis is regulated in both cases by their own genomic promoters. These strains were grown in rich medium to mid-exponential phase, subjected to a thermal stress, and the corresponding extracts obtained. Thereafter, Tps1p–GST and Ntp1p–Ha6H were immunoprecipitated with anti-GST and anti-Ha antibodies respectively. As shown in Figure 5, irrespective of the changed residue, the Ntp1p–Ha6H band was clearly detected with anti-Ha antibodies in the complexes obtained from Tps1p–GST immunoprecipitation (Figure 5A, lanes 2–6), whereas Tps1p–GST was visible with anti-GST antibodies after Ntp1p–Ha6H immunoprecipitation (Figure 5B, lanes 2–6). The

Figure 6 Expression, purification and Ca²⁺ binding of Ntp1p in mutants altered in the putative calcium-binding motif

(A) SDS/PAGE analysis of total proteins from IPTG-induced *E. coli* cultures expressing different versions of Ntp1p–Ha6H mutated in amino acid residues of the Ca²⁺-binding motif, and after Ni²⁺/nitroacetic acid (NTA) purification. Lane 1, Ntp1p–Ha6H; lane 2, Ntp1p–D97L–Ha6H; lane 3, Ntp1p–R100L–Ha6H; lane 4, Ntp1p–I104L–Ha6H; lane 5, Ntp1p–D108L–Ha6H; lane M, molecular mass markers. (B) The various proteins were transferred to a nitrocellulose membrane and incubated with anti-Ha antibodies. Lane C, purified Ntp1p–Ha6H from *S. pombe*. (C) Calcium overlay assay of Ntp1p. Samples of 10 µg each of purified Ntp1p–Ha6H wild-type (WT) and mutant proteins were dot-blotted on to a nitrocellulose membrane and incubated with ⁴⁵Ca²⁺ Cl₂ (see the Experimental section). Positive calcium-binding proteins included calmodulin (4 µg) and calpain (10 µg), whereas BSA (10 µg) and alcohol dehydrogenase (ADH; 10 µg) were employed as negative controls. The same membrane was stained with Ponceau S as a loading control.

existence of the Ntp1p–Tps1p interaction was observed not only during heat stress, but also in growing cells and in those under osmotic stress (results not shown). We conclude that mutations in the putative EF-hand motif do not interfere with the ability of Ntp1p to associate *in vivo* with Tps1p.

Ca²⁺ binding in neutral trehalases mutated in different residues of the putative calcium-binding motif

Results obtained thus far strongly suggested the occurrence in fission yeast of direct regulation of neutral trehalase activation by calcium ions through the putative bivalent ion-binding motif. We next examined the ability of the different Ntp1p variants to effectively bind Ca²⁺. To this end, we expressed recombinant versions of Ntp1p (control and mutants D97L, R100L, I104L and D108L) tagged with Ha6H at the C-terminus by employing the pET expression system in *E. coli* (see the Experimental section). After purification by affinity chromatography with Ni²⁺ beads (Figures 6A and 6B), identical amounts of Ntp1p variants were blotted on to a nitrocellulose membrane and subjected to a ⁴⁵Ca²⁺ overlay assay [34]. This technique has been shown to be particularly useful for identification of proteins containing EF-hand Ca²⁺-binding sites, such as calpain, calmodulin, calcineurin B, troponin C and calxectin ([37] and references therein). As shown in Figure 6(C), both non-mutated Ntp1p and the mutant I104L were able to bind ⁴⁵Ca²⁺ to an extent similar to calpain and less effectively than calmodulin, whereas mutant R100L bound

Figure 7 Analysis of Ntp1p complexes by gel filtration

High-speed supernatants of extracts from heat-shocked strains N1 (control; wild type Ntp1p-Ha6H) and N7 (Ntp1p-D97L-Ha6H), together with an extract from strain N1 treated with 10 mM EDTA, were size fractionated by gel filtration through a Superdex-200 column. Proteins in 100 μ l of each fraction were concentrated by trichloroacetic acid precipitation, separated by SDS/PAGE, transferred to nitrocellulose and incubated with anti-Ha antibodies. The asterisks denote the fractions with maximal trehalase activity in extracts from control strain N1.

the cation weakly. Notably, mutants D97L and D108L, which when expressed in *S. pombe* do not show enzyme activation, were unable to bind $^{45}\text{Ca}^{2+}$ (Figure 6C). These observations confirm that *S. pombe* neutral trehalase is a Ca^{2+} -binding protein. Furthermore, the results indicate that the aspartic acid residues at positions 97 and 108 in the enzyme, which are critical for trehalase activation, are directly involved in cation binding. Hence the capacity of neutral trehalase to be activated by different stressing conditions appears to correlate with its ability to bind calcium ions.

Effect of Ca^{2+} binding on the oligomerization state of Ntp1p

As deduced from gel filtration experiments, the active form of Ntp1p in *S. pombe* corresponds to free homodimers or homotrimers [15]. On the other hand, neutral trehalase mutants D97L and D108L, which fail to bind Ca^{2+} (Figure 6) or to undergo enzyme activation (Figure 3), are still able to interact with Tps1p (Figure 5). These results led us to consider that calcium binding might be an essential requirement for Ntp1p self-association, as a step prior to enzyme activation. To resolve this question, *S. pombe* strains N1 (wild-type Ntp1p) and N7 (mutant D97L) were cultured in YES medium to mid-exponential phase, subjected to a heat shock, and the corresponding cell extracts analysed under native conditions by FPLC. Moreover, a cell extract from heat-shocked cells of strain N1 was pretreated with 10 mM EDTA and subjected to the same procedure. The proteins eluted from the column in the different fractions were precipitated with trichloroacetic acid, resolved by SDS/PAGE, blotted, and subsequently analysed by Western blot using anti-Ha antibodies. The results revealed a differential pattern of elution for Ntp1p in each case (Figure 7). Ntp1p from control strain N1 was detected mainly in fractions 14 and 15, which is consistent with the previously described elution profile for the dimeric or trimeric active form of the enzyme [15]. However, when the same cell extract was pretreated with EDTA, the presence of which drastically lowers trehalase activity (Table 3), most Ntp1p was present in fractions 17 and 18, which correspond to monomeric forms of the protein (approximate molecular mass 84 kDa). Finally, the elution pattern of mutated Ntp1p from strain N7 was intermediate, with Ntp1p showing a clear displacement to lower molecular masses (fractions 15–17) that was not as dramatic as that observed in EDTA-treated extracts (Figure 7). As a whole, these results favour the idea that the alterations in the aspartic acid residues

that are critical for both Ntp1p calcium binding and enzyme activation also affect the oligomerization state of *S. pombe* neutral trehalase.

DISCUSSION

Unlike what has been observed previously for neutral trehalases from *Saccharomyces cerevisiae* and *Kluyveromyces lactis* [14,16], the addition of Ca^{2+} over a wide concentration range to cell extracts of *S. pombe* did not enhance trehalase activity. Remarkably, however, enzyme activity clearly decreased in the presence of Ca^{2+} -chelating agents (Table 3), suggesting that Ca^{2+} might be an essential component of the active form of Ntp1p in the fission yeast. Addition of further Ca^{2+} to enzyme preparations treated with chelating agents did not allow recovery of trehalase, but in fact inhibited the remaining activity (Table 3). A possible explanation for this additional decrease is that the trehalase of *S. pombe*, similar to trehalases from other organisms, is inhibited by neutral salts at unphysiological concentrations [26]. It could be argued that a salt effect in cell extracts might prevent the detection of both activation of trehalase by Ca^{2+} and recovery of enzyme activity in attempts to overcome Ca^{2+} sequestration by chelating agents. However, we also failed to show a significant effect of Ca^{2+} in enzyme samples after desalting trehalase preparations by gel filtration chromatography. These results unveil a major structural role for this ion, and suggest that removal of Ca^{2+} from active Ntp1p presumably promotes a stable conformational change that renders *in vitro* re-activation impossible. Hence the lack of direct activation *in vitro* by Ca^{2+} and the simultaneous requirement for this cation appears to be a distinct property of the neutral trehalase of *S. pombe*. The different trehalase activities remaining in samples treated with EDTA or EGTA might reflect the effects of bivalent cations other than Ca^{2+} . In this respect, it has been reported that Mn^{2+} has an effect similar to that of Ca^{2+} on trehalases from several yeasts, so that the Ca^{2+} function conferring enzyme activity might be supplied in part by adaptation of Mn^{2+} as well [14,16].

The observation that neutral trehalases from *Saccharomyces cerevisiae* and *K. lactis* contain a putative EF-like Ca^{2+} -binding sequence was first made by Amaral et al. [16], and subsequently extended to neutral trehalases from other yeasts and filamentous fungi, including *S. pombe*, *Candida albicans*, *Aspergillus nidulans* and *Neurospora crassa* [17,18,20]. More recent evidence indicates that this binding site is also present in the neutral trehalases from the insect pathogenic fungus *Metarhizium anisopliae* and the rice blast fungus *Magnaporthe grisea* [38,39]. However, no functional role has been demonstrated for these sequences. In many known EF-hand-containing proteins, such as calmodulin, these motifs are typically found in pairs that display co-operative binding for the cation. When the Ca^{2+} -binding conserved aspartate residues at positions 1 and 12 within the motif are altered, calcium binding is dramatically decreased or, in most cases, eliminated [23]. In addition, acidic residues at positions 3, 5 and 9 also play a significant role in the co-ordination of calcium ions, since changes in these and other neighbouring structural residues may lead to decreased affinity [40,41]. Ntp1p from *S. pombe*, and other fungal neutral trehalases, exhibit a single canonical EF-like motif, with aspartic residues appearing only at positions 1, 3 and 12 within the site (positions 97, 99 and 108 of the Ntp1p amino acid sequence). However, we have been able to show that Ca^{2+} is bound effectively by Ntp1p of *S. pombe* at a level comparable to that of other Ca^{2+} -binding proteins such as calpain, although much less than that of calmodulin (Figure 6). This represents the first physical evidence for a direct interaction between this ion and trehalases. Indeed, Ntp1p accommodates

Ca^{2+} by binding through aspartic acid residues in the motif, since single point mutations in such residues induce dramatic changes in the Ca^{2+} -binding properties of the trehalase. Moreover, changes in the affinity of the trehalase for Ca^{2+} result in a substantial loss of enzyme activity under stress conditions that otherwise induce strong trehalase activation and a concomitant increase in the level of the trehalose pool (Figures 3 and 4). Altogether, these results support the hypothesis that Ca^{2+} influences trehalase activity by forming part of the framework of the active enzyme. This conclusion is further strengthened by the fact that alterations in the Ca^{2+} -binding site result in a defect in the oligomeric assembly of Ntp1p molecules which is associated with reduced trehalase activity [15]. Although the effects of blocking the EF-like consensus site for Ca^{2+} on trehalase and those derived from the seizure of Ca^{2+} by chelating agents are not identical, they nevertheless lead to similar outcomes. Both processes result in decreased trehalase activity and a simultaneous shift in the elution pattern that reveals the induction of altered oligomerization forms of the Ntp1p enzyme not corresponding to native dimers or trimers (Figure 7). This is analogous to the effect observed for subunit aggregation of some Ca^{2+} -binding proteins, whose Ca^{2+} -dependent association can be reversed by EDTA [25].

The relevance of the above data and its implications for the regulation of trehalase *in vivo* are intriguing. Our results indicate that Ca^{2+} is needed not only for trehalase activity (Table 3), but also for full activation of the enzyme under stress conditions (Figure 3). Current evidence supports the view that the activity of fungal neutral trehalases results from a prior activation step, consisting of cAMP-dependent phosphorylation of the enzyme, which may be triggered *in vivo* by metabolic changes or stressing conditions and mimicked *in vitro* by mixtures containing ATP, PKA and cAMP [1,12,42–45]. In contrast with trehalases from other yeasts, activation of *S. pombe* trehalase has not been achieved *in vitro*, although numerous results suggest that phosphorylation of the enzyme does occur *in vivo* [29,46–49]. On the other hand, similar to that from *Saccharomyces cerevisiae*, the active form of trehalase in *S. pombe* represents dimers or trimers of Ntp1p [14,15]. Therefore the need for Ca^{2+} could be a prerequisite for *in vivo* Ntp1p activation induced by both glucose and stressing conditions; alternatively, Ntp1p might be first phosphorylated, but unable to form proper oligomers in the absence of Ca^{2+} . Since determination of activation *in vivo* relies on measuring the trehalase activity of already phosphorylated molecules, any effect of Ca^{2+} on activity precludes estimation of a separate effect on the prior activation process. For this reason, it may be difficult to distinguish with certainty whether Ca^{2+} is important for either correct activation by phosphorylation or appropriate oligomerization folding that leads to the catalytically active form of Ntp1p. However, considering the influence of Ca^{2+} and chelating agents on trehalase assays *in vitro*, where only Ntp1p molecules already modified by activation and properly oligomerized exhibit enzyme activity, it seems reasonable to suspect that the main effect of Ca^{2+} removal is on the phosphorylated form of trehalase.

Interaction between Ntp1p and Tps1p is required in *S. pombe* for trehalase activation induced by some stresses [5,36]. Thus we considered that this might be another potential point for a Ca^{2+} -mediated effect on trehalase, particularly because formation of Ntp1p–Tps1p complexes has been demonstrated *in vivo* in the fission yeast [15]. However, when we focused on this issue, the results showed that mutant strains altered in amino acid residues within the Ca^{2+} -binding site still displayed the ability for Ntp1p–Tps1p association (Figure 5).

It has been postulated that trehalase activation by heat shock in *Saccharomyces cerevisiae* requires the prior removal of an endo-

genous inhibitor protein that prevents trehalase phosphorylation by PKA [50]. A model for the regulation of neutral trehalase by Ca^{2+} has been proposed in this yeast that involves the phosphorylation of this trehalase-associated protein by Ca^{2+} /calmodulin-dependent protein kinase to induce its dissociation from the trehalase enzyme, thus exposing the sites of the enzyme that are susceptible to phosphorylation by PKA [51]. Accordingly, the role of Ca^{2+} in the modulation of neutral trehalase activity in *Saccharomyces cerevisiae* appears to rely upon a synergy between the ion and the cAMP signalling pathway that regulates trehalase activation. In contrast, our interpretation of the effect of Ca^{2+} on Ntp1p from *S. pombe* is much simpler, and based on a direct structural relationship between Ca^{2+} and the Ntp1p enzyme itself. Consistent with this, we have demonstrated that structural integrity of the binding site for Ca^{2+} is required for trehalase activation upon stress and that, independent of the nature of the activation mechanism, Ca^{2+} binding is required for the normal catalytic activity of the enzyme through the homomeric association of Ntp1p molecules. Taking into account the conserved nature of the Ca^{2+} -binding motif, it is possible that neutral trehalase activity is modulated by Ca^{2+} switching in other fungal systems as well. However, specific requirements may be additionally involved, since the *in vitro* response of trehalase to the addition of Ca^{2+} is not identical in fission and budding yeasts. It is likely that the Ca^{2+} -induced formation of trehalase oligomers is necessary, but not sufficient, to bring about trehalase activation. This interpretation correlates with the observation that, unlike for *Saccharomyces cerevisiae* neutral trehalase, phosphorylation of Ntp1p has not yet been achieved *in vitro*, and may be also dependent on interactions with other enzymes related to trehalose metabolism *in vivo* [15].

We are indebted to S. Pabmanabhan for technical assistance. A.F., V.P. and M.M. are predoctoral fellows of the Universidad de Murcia, Fundación Séneca (Región de Murcia), and Formación del Profesorado Universitario (FPU) respectively. This work was supported in part by grant BMC 2002-01104 from MCYT, Spain.

REFERENCES

- Nwaka, S. and Holzer, H. (1998) Molecular biology of trehalose and the trehalases in the yeast *Saccharomyces cerevisiae*. *Prog. Nucleic Acid Res. Mol. Biol.* **58**, 197–237
- Thevelein, J. M. (1984) Regulation of trehalose mobilization in fungi. *Microbiol. Rev.* **48**, 42–59
- De Virgilio, C., Hottiger, T., Dominguez, J., Boller, T. and Wiemken, A. (1994) The role of trehalose synthesis for the acquisition of thermotolerance in yeast I. Genetic evidence that trehalose is a thermoprotectant. *Eur. J. Biochem.* **219**, 179–186
- Hottiger, T., De Virgilio, C., Hall, M. N., Boller, T. and Wiemken, A. (1994) The role of trehalose synthesis for the acquisition of thermotolerance in yeast II. Physiological concentrations of trehalose increase the thermal stability of proteins *in vitro*. *Eur. J. Biochem.* **219**, 187–193
- Ribeiro, M. J. S., Reinders, A., Boller, T., Wiemken, A. and De Virgilio, C. (1997) Trehalose synthesis is important for the acquisition of thermotolerance in *Schizosaccharomyces pombe*. *Mol. Microbiol.* **25**, 571–581
- Soto, T., Fernández, J., Vicente-Soler, J., Cansado, J. and Gacto, M. (1999) Accumulation of trehalose by overexpression of tps1, coding for trehalose-6-phosphate synthase, causes increased resistance to multiple stresses in the fission yeast *Schizosaccharomyces pombe*. *Appl. Environ. Microbiol.* **65**, 2020–2024
- Cansado, J., Soto, T., Fernández, J., Vicente-Soler, J. and Gacto, M. (1998) Characterization of mutants devoid of neutral trehalase activity in the fission yeast *Schizosaccharomyces pombe*: partial protection from heat shock and high-salt stress. *J. Bacteriol.* **180**, 1342–1345
- Clegg, J. S. (1985) The physical properties and metallic status of *Artemia* cysts at low water contents. The “water replacement hypothesis”. In *Membranes, Metabolism and Dry Organisms* (Leopold, A. C., ed.), pp. 169–187. Cornell University Press, Ithaca
- Singer, M. A. and Lindquist, S. (1998) Multiple effects of trehalose on protein folding *in vitro* and *in vivo*. *Mol. Cell* **1**, 639–648
- Nwaka, S., Mechler, B. and Holzer, H. (1996) Deletion of the *ATH1* gene in *Saccharomyces cerevisiae* prevents growth on trehalose. *FEBS Lett.* **386**, 235–238

- 11 Kopp, M., Müller, H. and Holzer, H. (1993) Molecular analysis of the neutral trehalase gene from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **268**, 4766–4774
- 12 Thevelein, J. M. (1988) Regulation of trehalase activity by phosphorylation-dephosphorylation during developmental transitions in fungi. *Exp. Mycol.* **12**, 1–7
- 13 Thevelein, J. M. (1996) Regulation of trehalose metabolism and its relevance to cell growth and function. In *The Mycota III, Biochemistry and Molecular Biology* (Brambl, R. and Marzluf, G.A., eds.), pp. 395–414, Springer-Verlag, Heidelberg
- 14 Londesborough, J. and Varimo, K. (1984) Characterization of two trehalases in baker's yeast. *Biochem. J.* **219**, 511–518
- 15 Soto, T., Franco, A., Padmanabhan, S., Vicente-Soler, J., Cansado, J. and Gacto, M. (2002) Molecular interaction of neutral trehalase with other enzymes of trehalose metabolism in the fission yeast *Schizosaccharomyces pombe*. *Eur. J. Biochem.* **269**, 3847–3855
- 16 Amaral, F. C., Van Dijck, P., Nicoli, J. R. and Thevelein, J. M. (1997) Molecular cloning of the neutral trehalase gene from *Kluyveromyces lactis* and the distinction between neutral and acid trehalases. *Arch. Microbiol.* **167**, 202–208
- 17 d'Enfert, C., Bonini, B. N., Zapella, P. D. A., Fontaine, T., da Silva, A. M. and Terenzi, H. F. (1999) Neutral trehalases catalyse intracellular trehalose breakdown in the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*. *Mol. Microbiol.* **32**, 471–483
- 18 Eck, R., Bergmann, C., Ziegelbauer, K., Schonfeld, W. and Kunkel, W. (1997) A neutral trehalase gene from *Candida albicans*: molecular cloning, characterization and disruption. *Microbiology* **143**, 3747–3756
- 19 Nwaka, S., Kopp, M. and Holzer, H. (1995) Expression and function of the trehalase genes *NTH1* and *YBR0106* in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **270**, 10193–10198
- 20 Soto, T., Fernández, J., Domínguez, A., Vicente-Soler, J., Cansado, J. and Gacto, M. (1998) Analysis of *npt1+* gene, encoding neutral trehalase in the fission yeast *Schizosaccharomyces pombe*. *Biochim. Biophys. Acta* **1443**, 225–229
- 21 Geiser, G. R., Van Tuinen, D., Brokerhoff, S. E., Neff, M. M. and Davis, T. N. (1999) Can calmodulin function without binding calcium? *Cell* **65**, 949–959
- 22 Wera, S., De Schrijver, E., Geyskens, I., Nwaka, S. and Thevelein, J. M. (1999) Opposite roles of trehalase activity in heat-shock recovery and heat-shock survival in *Saccharomyces cerevisiae*. *Biochem. J.* **343**, 621–626
- 23 Rashidi, H. H., Bauer, M., Patterson, J. and Smith, D. W. (1999) Sequence motifs determine structure and Ca^{++} -binding by EF-hand proteins. *J. Mol. Microbiol. Biotechnol.* **1**, 175–182
- 24 Nakashima, K., Ishida, H., Ohki, S. Y., Hikichi, K. and Yazawa, M. (1999) Calcium binding induces interaction between the N- and C-terminal domains of yeast calmodulin and modulates its overall conformation. *Biochemistry* **38**, 98–104
- 25 Pal, G. P., Elce, J. S. and Jia, Z. (2001) Dissociation and aggregation of calpain in the presence of calcium. *J. Biol. Chem.* **276**, 47233–47238
- 26 Moreno, S., Klar, A. and Nurse, P. (1991) Molecular genetic analysis of the fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol.* **194**, 795–823
- 27 Boeke, J. D., La Croute, F. and Fink, G. R. (1984) A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol. Gen. Genet.* **197**, 345–346
- 28 Higuchi, R., Krummel, B. and Saiki, R. K. (1988) A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res.* **16**, 7351–7367
- 29 Carrillo, D., Vicente-Soler, J. and Gacto, M. (1994) Cyclic AMP signalling pathway and trehalase activation in the fission yeast *Schizosaccharomyces pombe*. *Microbiology* **140**, 1467–1472
- 30 Fernández, J., Soto, T., Vicente-Soler, J., Cansado, J. and Gacto, M. (1997) Heat-shock response in *Schizosaccharomyces pombe* cells lacking cyclic AMP-dependent phosphorylation. *Curr. Genet.* **31**, 112–118
- 31 Fernández, J., Soto, T., Vicente-Soler, J., Cansado, J. and Gacto, M. (1997) Osmo-stress-induced changes in neutral trehalase activity of the fission yeast *Schizosaccharomyces pombe*. *Biochim. Biophys. Acta* **1357**, 41–48
- 32 Beltrán, F. F., Castillo, R., Vicente-Soler, J., Cansado, J. and Gacto, M. (2000) Role for trehalase during germination of spores in the fission yeast *Schizosaccharomyces pombe*. *FEMS Microbiol. Lett.* **193**, 117–121
- 33 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 34 Maruyama, K., Mikawa, T. and Ebashi, S. (1984) Detection of calcium binding proteins by ^{45}Ca autoradiography on nitrocellulose membrane after sodium dodecyl sulfate gel electrophoresis. *J. Biochem. (Tokyo)* **95**, 511–519
- 35 Mitsuho, I. (1996) Calcium binding and conformational response in EF-hand proteins. *Trends Biochem. Sci.* **21**, 4–17
- 36 Cansado, J., Vicente-Soler, J., Soto, T., Fernández, J. and Gacto, M. (1998) Trehalose-6P synthase is essential for trehalase activation triggered by glucose, nitrogen source or heat shock, but not by osmotic stress in *Schizosaccharomyces pombe*. *Biochim. Biophys. Acta* **1381**, 271–278
- 37 Bian, S., Favre, I. and Moczydlowski, E. (2001) Ca^{2+} -binding activity of a COOH-terminal fragment of the *Drosophila* BK channel involved in Ca^{2+} -dependent activation. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 4776–4781
- 38 Foster, A. J., Jenkinson, J. M. and Talbot, N. J. (2003) Trehalose synthesis and metabolism are required at different stages of plant infection by *Magnaporthe grisea*. *EMBO J.* **22**, 225–235
- 39 Xia, Y., Gao, M., Clarkson, J. and Charnley, A. (2003) Molecular cloning, characterization, and expression of a neutral trehalase from the insect pathogenic fungus *Metarhizium anisopliae*. *J. Invertebr. Pathol.* **80**, 127–137
- 40 Chandra, M., McCubbin, W. D., Oikawa, K., Kay, C. M. and Smillie, L. B. (1994) Ca^{++} , Mg^{++} and troponin I inhibitory peptide binding to a Phe-154 to Trp mutant in chicken skeletal muscle troponin C. *Biochemistry* **33**, 2961–2969
- 41 Starovasnik, M. A., Su, D. R., Beckingham, K. and Klevit, R. E. (1992) A series of point mutations reveal interactions between the calcium-binding sites of calmodulin. *Protein Sci.* **1**, 245–254
- 42 App, H. and Holzer, H. (1989) Purification and characterization of neutral trehalase from the yeast ABY51 mutant. *J. Biol. Chem.* **264**, 17583–17588
- 43 Thevelein, J. M. and Beullens, M. (1985) Cyclic AMP and the stimulation of trehalase activity in the yeast *Saccharomyces cerevisiae* by carbon source, nitrogen sources and inhibitors of protein synthesis. *J. Gen. Microbiol.* **131**, 3199–3209
- 44 Uno, I., Matsumoto, K., Adachi, K. and Ishikawa, T. (1983) Genetic and biochemical evidence that trehalase is a substrate of cAMP-dependent protein kinase in yeast. *J. Biol. Chem.* **258**, 10867–10872
- 45 Vanhalewyn, M., Dumortier, F., Debat, G., Colombo, S., Ma, P., Winderickx, J., Van Dijck, P. and Thevelein, J. M. (1999) A mutation in *Saccharomyces cerevisiae* adenylate cyclase, Cyr1K1876M, specifically affects glucose- and acidification-induced cAMP signalling and not the basal cAMP level. *Mol. Microbiol.* **33**, 363–376
- 46 Soto, T., Fernández, J., Cansado, J., Vicente-Soler, J. and Gacto, M. (1995) Nitrogen-source-induced activation of neutral trehalase in *Schizosaccharomyces pombe* and *Pachysolen tannophilus*: role of cAMP as second messenger. *FEMS Microbiol. Lett.* **132**, 229–232
- 47 Soto, T., Fernández, J., Cansado, J., Vicente-Soler, J. and Gacto, M. (1995) Glucose-induced, cyclic-AMP-independent signalling pathway for activation of neutral trehalase in the fission yeast *Schizosaccharomyces pombe*. *Microbiology* **141**, 2665–2671
- 48 Soto, T., Fernández, J., Cansado, J., Vicente-Soler, J. and Gacto, M. (1995) Activation of neutral trehalase by glucose and nitrogen source in *Schizosaccharomyces pombe* strains deficient in cAMP-dependent protein kinase activity. *FEBS Lett.* **367**, 263–266
- 49 Soto, T., Fernández, J., Cansado, J., Vicente-Soler, J. and Gacto, M. (1997) Protein kinase Sck1 is involved in trehalase activation by glucose and nitrogen source in the fission yeast *Schizosaccharomyces pombe*. *Microbiology* **143**, 2457–2463
- 50 De Mesquita, J. F., Paschoalin, W. M. F. and Panek, A. D. (1997) Modulation of trehalase activity in *Saccharomyces cerevisiae* by an intrinsic protein. *Biochim. Biophys. Acta* **1334**, 233–239
- 51 Souza, A. C., De Mesquita, J. F., Panek, A. D., Silva, J. T. and Paschoalin, V. M. F. (2002) Evidence for a modulation of neutral trehalase activity by Ca^{2+} and cAMP signaling pathways in *Saccharomyces cerevisiae*. *Braz. J. Med. Biol. Res.* **35**, 11–16

Received 4 June 2003/18 August 2003; accepted 28 August 2003

Published as BJ Immediate Publication 28 August 2003, DOI 10.1042/BJ20030825